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Comparative susceptibilities of insect cell lines to infection by the occlusion-body derived phenotype of baculoviruses

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Abstract

Twelve insect cell lines from six species were tested for susceptibility to baculovirus infection by occlusion-derived virus (ODV) phenotype through the use of a typical endpoint assay procedure. ODV from three nucleopolyhedroviruses were prepared by alkali treatment (sodium carbonate) of occlusion bodies (OBs) and the virus preparations were titered on various cell lines. More than a four-log difference was realized for each of theses viruses between the various cell lines. The TN368 line from *Trichoplusia ni* was only marginally susceptible to ODV from each virus, showing only 3–6 infectious units (IU) per million OBs while the gypsy moth line, LdEp was most susceptible, realizing more than 100,000 IU/million OBs. The other lines tested showed various levels of susceptibility between these two extremes and also varied between the three viruses tested. In additional tests, the ODV were treated with trypsin prior to application to the cells. With most cell lines, this treatment increased the infectivity of each virus by 2–10-fold. Exceptions to this trend included the gypsy moth LdEp line, on which the trypsinized ODV from two of the viruses were slightly less infectious than each virus without trypsin, and the TN-368 line, on which the trypsinized ODV was 5000–75,000 times more infectious. The variable results of trypsinized virus on the different lines are probably due to the levels of endogenous protease activity in the various lines, but the mode of action of the trypsin has not been elucidated. Ultimately, the variable response of cell lines to ODV of different viruses, and the variable effects of trypsin on the ODV may lead to an improved understanding of the infection process of this virus phenotype as well as factors relating to baculovirus host range.
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Keywords: Insect cell lines; Nucleopolyhedrovirus; AcMNPV; AfMNPV; AgMNPV; Occlusion bodies; Virus infectivity; Trypsin

1. Introduction

Insect cell lines have been used for studying viruses almost since the first continuous lines were established. Grace (1967) showed that the first moth cell lines, his *Antherea pernyii* lines, were susceptible to *Bombyx mori* nucleopolyhedrovirus (NPV). Subsequently, many baculoviruses have been replicated in a wide variety of lepidopteran cell lines (Granados and Hashimoto, 1989). Typically, researchers used hemolymph or whole body homogenates from diseased insects as the source of infectious material. Dougherty et al. (1975) and Ramoska (1976) determined the infectious material for NPVs in cell cultures was the nonoccluded form of virus (NOV) produced during budding of the nucleocapsid through the cytoplasmic membrane (thus they are also known as budded virus, BV). The BV form is also responsible for cell-to-cell transmission of the virus within the host larvae.

Alternatively, NPV virions that are occluded in the polyhedrin matrix are more stable in the environment and are responsible for transmission of the disease between individual insects. The infection process with the polyhedra (occlusion bodies, OBs) functions because the digestive tract contents of susceptible insects are at a high pH and the polyhedrin protein is soluble under such conditions. Thus, the infection process is initiated when a susceptible insect larva consumes some OBs occurring as a contaminant on its diet. When the OBs reach the alkaline conditions in the midgut, they are dissolved, releasing the enveloped virions. These fuse to the microvilli on the brush border of the midgut columnar cells to start the infection. Much of what is

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known about the infection process is based on morphological evidence although some data exist that the attachment of virions to the cell membrane is receptor mediated (Horton and Burand, 1993). Subsequently, the infection spreads via BV from the midgut cells to other tissues in the insect. In some cases, this occurs by the production of progeny viruses in the midgut cells that go on to infect secondary tissues (such as trachea, Engelhard et al., 1994) but suggestions have also been made that OB-derived virions (ODV) can directly bud from the midgut cells (Granados and Lawler, 1981). While molecular tools and systems such as brush border membranes can assist in delineating this process, the insect midgut is a difficult tissue on which to perform reproducible experiments.

In their natural state, OBs are not infectious to cell cultures because culture media are typically slightly acidic, thus precluding the release of virions from the polyhedrin matrix. ODV can be released from polyhedra by treatment with an alkaline solution, typically sodium carbonate. While these are infectious to cells in culture, infection is inefficient. For example, Vail et al. (1979) found that approximately 27,500 OBs were required per plaque forming unit. In a comparative study with BV and ODV, Volkman et al. (1976) estimated that BV is 1700–1900-fold more infectious than ODV to TN-368 cell cultures. More recently, McIntosh and Ignoffo (1988) found that proteinase treatment on the dissolved *Helicoverpa zea* NPV (HzSNPV) OBs improved infectivity and I found similar results using trypsin on ODV from *Lymantria dispar* NPV (LdMNPV) (Lynn, 1994). My previous study also showed that the positive effects of enzyme treatment were not universal with all cell lines. While trypsinized ODV (ODV-T) were 50 times more infectious to a gypsy moth fat body cell line (IPLB-LdFB) than virus that was not trypsinized, no appreciable change in infectivity was observed in trypsinized virus on three other gypsy moth lines. Confounding these results was the fact that an ovary-derived line (IPLB-Ld652Y) had a low level of susceptibility to ODV (slightly lower than ODV without trypsin in LdFB) and this level of infectivity for these cells was not improved with trypsin treatment, while the two embryo-derived lines tested in that study (IPLB-LdEIta and IPLB-LdEp) were slightly more susceptible to ODV than the fat body line was to ODV-T. In other words, Ld652Y was marginally susceptible to ODV, and this was not improved by trypsin; LdEIta and LdEp were much more highly susceptible but again, not improved by trypsin; and LdFB susceptibly was variable—low to ODV but much higher to ODV-T.

In the current experiments, I expanded these earlier studies to other virus/cell systems, notably some of the noctuid NPVs with wider host ranges than either LdMNPV or HzSNPV. I have recently shown 100-fold variable susceptibilities between the most susceptible and least susceptible of a dozen insect cell lines to BV from three noctuid NPVs, namely *Autographa californica* NPV (AcMNPV), *Anagrapha falcifera* NPV (AfMNPV), and *Anticarsia gemmatalis* NPV (AgMNPV) (Lynn, 2003). AfMNPV is generally considered a variant of AcMNPV (Federici and Hice, 1997) although with some in vivo host range differences (Hostetter and Puttler, 1991) while AgMNPV is more distantly related to the other two although still in the Group 1 NPVs (Zanotto et al., 1993).

In the current study, I have asked whether the susceptibility of these lines to ODV correlates to the results with BV. In addition, some of the additional tools available from the widely studied AcMNPV were used to examine some possible reasons for the variable trypsin effects observed with LdMNPV. Since some the gypsy moth lines used in my earlier study are susceptible to AcMNPV, these new studies will allow additional comparisons in how the trypsin effect on ODV is being manifested in cells with different viruses. Also, since trypsin does not universally increase the infectivity of ODV to all cell lines, or to an equivalent level in the cells on which it does increase ODV infectivity, these variable effects can provide tools to help delineate how the enzyme is producing its effects. Ultimately, the results from this study may provide an additional tool to help delineate the infection process by ODV.

2. Materials and methods

2.1. Cell Lines

Cell lines used in these studies and the media used for each are listed in Table 1. Each line was at high passage (over 200) and cultures were maintained in 25 cm^2 Greiner Cellstar tissue culture flasks (Frickenhausen, Germany) at 26 °C on a weekly subculture interval. Cell identity was confirmed by isozyme analysis.

2.2. Virus

The baculoviruses used in these studies were clones of AcMNPV (AcMNPV-pxh; Lynn, 2002), AfMNPV (AfMNPV-sf; Lynn, 2003), and AgMNPV (AgMNPV-ag3; Lynn, 2003). OB samples from each virus were prepared from infected cell cultures by centrifuging cells (250*g*) for 15 min, resuspending in sterile distilled water (sdH_2O), washing with 0.5% (w/v) sodium laurel sulfate (Sigma, St. Louis, MO), and rinsing three times with sdH_2O (centrifuging at 250*g* between each wash and rinse). The final pellet was adjusted to 1×10^8 OBs/ml sdH_2O and stored at 4 °C. The same virus preparations were used throughout this study although OB preparations made from other culture infections give similar results (data not shown). ODV were prepared from

Table 1
Cell lines used

Species	Designation	Passage level	Medium	Reference
Anticarsia gemmatalis	UFL-Ag286	480	TC-100-II	Sieburth and Maruniak (1988)
Heliothis virescens	IPLB-HvE1a	250	TC-100-II	Lynn and Shapiro (1998)
H. virescens	IPLB-HvE6a	260	TC-100-II	Lynn and Shapiro (1998)
H. virescens	IPLB-HvE6s	290	TC-100-II	Lynn and Shapiro (1998)
H. virescens	IPLB-HvT1	400	TNM-FH	Lynn et al. (1988)
Lymantria dispar	IPLB-LdEIta	690	Ex-Cell 400	Lynn et al. (1988)
L. dispar	IPLB-LdEp	560	Ex-Cell 400	Lynn et al. (1988)
Plutella xylostella	IPLB-PxE2	660	TC-100-II	Lynn (1989)
Spodoptera frugiperda	IPLB-Sf21AE	Very high	Ex-Cell 400	Vaughn et al. (1977)
Trichoplusia ni	TN-368	Very high	TNM-FH	Hink (1970)
T. ni	IAL-TND1	1090	IPL-52B-P	Lynn et al. (1982)
T. ni	IPLB-TNR[2]	840	TNM-FH	Rochford et al. (1984)

these as described previously (Lynn, 1994). OBs were treated for 30 min with 25 mM $NaCO_3$/50 mM NaCl followed by addition of an acidic salt solution to bring the sample back to near neutrality. An aliquot was diluted in modified TC100 medium (= ODV) while the remainder was treated with 0.1 mg/ml VMF trypsin (Worthington Biochemicals, Freehold, NJ) for 2 h before also being diluted in modified TC100 (= ODV-T). The fetal bovine serum (9% v/v) in modified TC100 acts to suppress further trypsin activity.

2.3. Endpoint assays

The procedure was similar to that used previously for titration of BV (Lynn, 2002). Cells of each line were counted in situ using a Nikon TMS inverted microscope fitted with a calibrated reticulated eyepiece. After counting, cells were suspended by trypsinization (HvE1a, HvE6a, and HvT1) or by flushing with medium from a transfer pipet (all other lines) and diluted in their respective medium to 1×10^5 cells/ml. Cells (5000 in 0.2 ml) were distributed into each well of a 96-well plate (Corning, Cambridge, MA). The virus samples (either ODV or ODV-T) were diluted through 10-fold serial dilutions in modified TC-100 to 10^{-6} of the original concentrations. Aliquots (10 μl) of each dilution were inoculated onto each of eight wells of the respective cell lines. The plates were sealed with masking tape and incubated at 22 °C.[1] Cells were examined for cytopathic effect (the presence of occlusion bodies in the nuclei) up through four weeks. The resulting data were analyzed by the Spearman–Kärber method (Lynn, 1992) to provide tissue culture infectious dose estimates for 50% of the wells ($TCID_{50}$) and then treated by one-way analysis of variance and Tukey's mean comparison test in the SigmaStat statistical program (version 2.0). The $TCID_{50}$ values were subsequently converted to infectious units per million OBs for presentation in the figures.

[1] This lower temperature was used because of my earlier findings that improved titers are obtained with some cell lines as compared with 27 °C (Lynn, 2002).

3. Results and discussion

Comparative susceptibilities of a dozen cell lines that were permissive for replication of AcMNPV, AfMNPV, and AgMNPV to ODV and ODV-T from these virus species are shown in Figs. 1A–C. Several points can be made from these results. First, the relative susceptibility of these cell lines varied by more than 20,000-fold between the least and most susceptible, and a similar range was seen with each of these noctuid multiple-embedded NPVs although variability was greatest with cells infected with AgMNPV (from less than 3 $TCID_{50}$-infectious units (IU) per million OBs for two of the *Trichoplusia ni* cell lines to over 800,000 IU/million OBs with the *A. gemmatalis* line). The effect of trypsinization on ODV was also demonstrated in these figures. For most cell lines, each of these viruses received a slight, but consistent, boost in the infectivity of one-half to one log increase. The largest difference between ODV and ODV-T infectivities was with Hink's TN-368 cabbage looper line. This line increased from 4 IU/million OBs (the lower threshold for this assay is 3 IU/million) to over 19,000 with the AcMNPV and from 6 IU/million OBs to over 460,000 with the AfMNPV, which were the lowest and highest values recorded with this virus. Alternatively, the IPLB-LdEp gypsy moth cell line showed a slight decrease in infectivity of these two viruses following trypsinization but it actually had the highest level of susceptibility to AcMNPV when treated with ODV of all the cell lines that were tested and was only slightly lower than ODV-T on TN-368.

Fig. 2 presents the same results as shown in Fig. 1 except only showing the ODV-T data and with the results for each of the three viruses grouped together for each cell line. This presentation revealed the relative susceptibility of the cell lines to the ODV-T of each

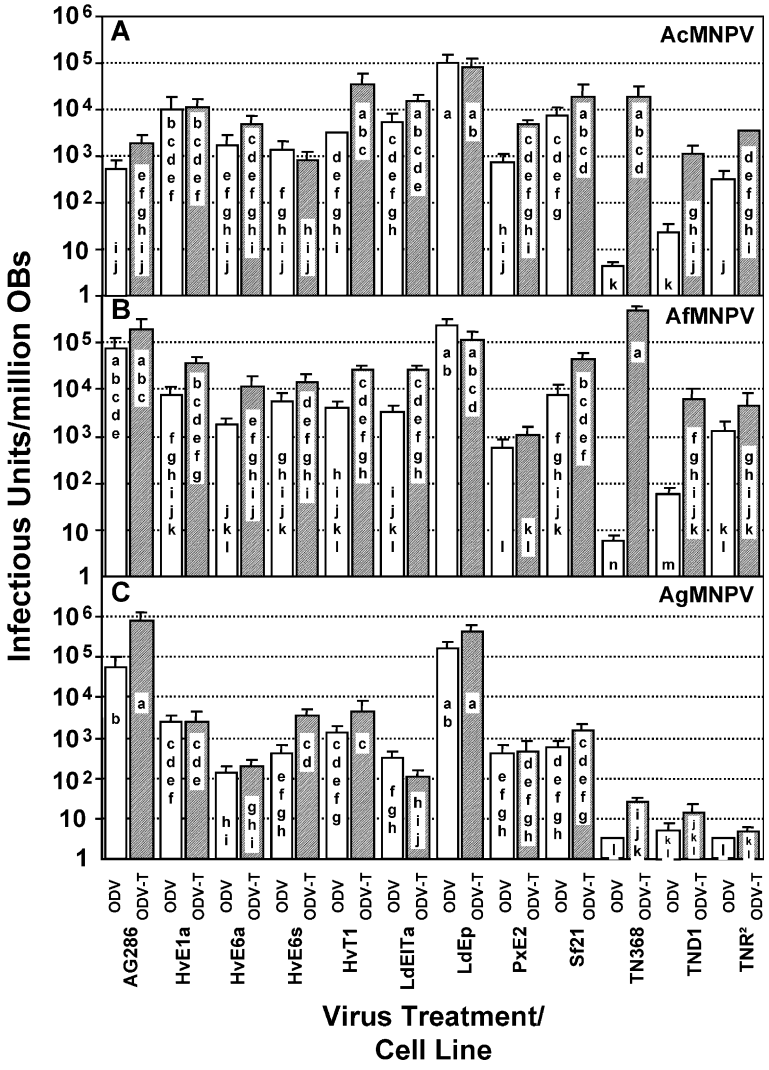

Fig. 1. Results of endpoint assays of occlusion-derived virus on insect cell lines. Titers were determined based on the presence of occlusion bodies in the nuclei after four weeks at 22 °C. Each bar is the titer of the virus on the cell line listed on the *x*-axis normalized to 1×10^6 occlusion bodies. White bars are titers on occlusion-derived virus, shaded bars are titers on trypsinized occlusion-derived virus. Titers and the standard errors (represented by error bars on the graph) were calculated by Spearman–Kärber (Lynn, 1992). Bars within the same graph labeled with the same lowercase letter were not statistically different as determined by Tukey's mean comparison test ($P < 0.05$). (A) AcMNPV, (B) AfMNPV, and (C) AgMNPV.

virus. Additionally, by averaging the $TCID_{50}$ data from all the cell lines for each virus, the variable infectivity of the three viruses was revealed by the resulting standard deviations. The amount of variability was similar between AcMNPV and AfMNPV (standard deviations of the log, SD_{log} of 0.61 and 0.72, respectively) but much greater with AgMNPV ($SD_{log} = 1.63$). Two factors explain the greater variability with AgMNPV. The homologous *A. gemmatalis* cell line (Ag286) was about 2.6 logs more susceptible to AgMNPV than it was to AcMNPV while the cabbage looper lines (TN368, TND1, and TN-R^2) had the opposite response to these two viruses (1.9–2.9 logs greater susceptibilities to AcMNPV than AgMNPV). Neither of these differential responses is surprising since previous studies have established that the AcMNPV is highly infectious to the cabbage looper (Vail and Jay, 1973) but less so to the velvetbean caterpillar (Moscardi, 1999) while the reverse is true for AgMNPV (highly infectious to *A. gemmatalis*, less so to *T. ni*) (Grasela and McIntosh, 1998).

The results obtained with the gypsy moth LdEp cell line may be the most surprising in these studies. None of the viruses used in these studies shows an appreciable level of infectivity to gypsy moth larvae (Martin Shapiro, personal communication) yet this line was one of the most susceptible to each of these viruses. Additionally, as I previously reported with ODV from the gypsy moth NPV on this cell line (Lynn, 1994), trypsin treatment did not greatly improve infectivity of any of the viruses on the LdEp cell line. Testing with a trypsin assay agent (TAME) was inconclusive, but the possibility exists that the viruses were not improved by trypsin on the LdEp cell line

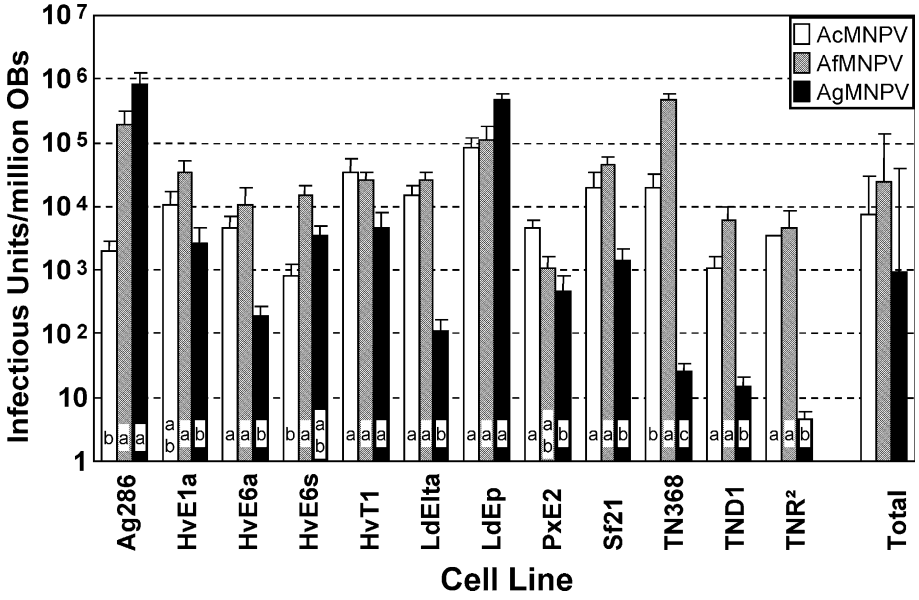

Fig. 2. Comparison of trypsinized occlusion-derived virus titers obtained on the cell lines listed on the *x*-axis. The final set of bars (labeled Total) are the means and standard deviations of the titers of each virus on all 12 cell lines. Data are as described in Fig. 1 except the white bars are the results AcMNPV, shaded bars are AfMNPV, and black bars are AgMNPV. The same lowercase letters on each bar indicates no significant difference as determined by Tukey's mean comparison test ($P < 0.05$) in titers between the viruses on that specific cell line.

because these cells were able to "activate" ODV with their endogenous proteases. As detailed in Table 1, different media were used on the various cell lines and this could be effecting these results. The medium of choice was the one that consistently provides the best growth of each line in this laboratory. Thus, nine lines were grown on three media formulations that were supplemented with 9% v/v fetal bovine serum (FBS) while the other three lines (LdEp, LdEIta, and Sf21) were on a serum-free formulation. Since FBS naturally contains trypsin inhibitors, endogenous trypsin in cells grown in the serum-containing formulations may be suppressed to some extent. While this could explain some of the differential results between ODV and ODV-T on various lines, it is not absolute since ODV tested on some cell lines in the presence of FBS had fairly high levels of infectivity (for example, AfMNPV on Ag286) while ODV-T from each virus was still 2–5-fold more infectious than ODV on the Sf21 cell line in serum-free medium.

Regardless of the action of endogenous proteases, the question of how the trypsin is improving the infectivity of ODV remains. One possibility is that the enzyme acts to create an active receptor ligand by cleaving an ODV membrane-associated protein. Horton and Burand (1993) showed that specific binding sites exists for ODV on host cells, and Faulkner et al. (1997) indicated that a 74 kDa protein on the ODV envelope is essential for per os infection of OBs, leading one to conclude that the p74 protein may act as the ODV attachment factor during the infection process. Surprisingly, I saw no reduction in infectivity in preliminary tests with ODV and ODV-T prepared from a p74-deletion virus (AcLP4, Faulkner et al., 1997) as compared with wild-type AcMNPV virus in the endpoint assays (data not shown). This suggests that p74 is not an essential protein for infections of cell cultures with ODV. While this seems contradictory to the possible role of p74 in ODV attachment, it is consistent with the previously reported fact that insect larvae can be infected by intrahemocoelic injection of the p74-deletion ODV (Faulkner et al., 1997). While this does not help answer the role of trypsin in the infection process, it does suggest the infection process by ODV in cell culture has some difference from what occurs in the midgut of susceptible larvae or, at the very least, that the phase of the larval infection process in which p74 is important does not exist in the cell culture infection process.

Even so, the infection process with ODV in cell culture is clearly different from that with BV. Table 2 lists the cell lines ranked in order of susceptibility to ODV-T for each virus (left columns) and in order of susceptibility to BV (right columns; data from Lynn, 2003). While the susceptibility of the two viral phenotypes from AgMNPV correlated fairly well (with a couple of exceptions; LdEp was more susceptible to ODV and the *Plutella xylostella* PxE2 line was more susceptible to BV), this was not the case for the other two viruses. For example with AcMNPV, the four least susceptible lines to ODV-T (TNR^2, Ag286, TND1, and HvE6s) were among the top five most susceptible to BV and two of the most susceptible to ODV-T (TN-368 and LdEp) were the least susceptible to BV.

While it is not surprising that differences would exist in cell line susceptibility between BV and ODV, the

Table 2
Ranks of cell line susceptibilities to occlusion-body derived virus and budded virus

Rank ODV-T (BV)	Cells	log(IU/10^6)	SE	Rank BV (ODV-T)	Cells	log($TCID_{50}$/ml)	SE
AcMNPV				AcMNPV			
1 (11)	LdEp	4.92	0.17	1 (9)	TNR[2]	9.42	0.13
2 (4)	HvT1	4.54	0.22	2 (10)	Ag286	8.75	0.13
3 (12)	TN368	4.29	0.21	3 (11)	TND1	8.42	0.08
3 (8)	Sf21	4.29	0.23	4 (2)	HvT1	8.00	0.16
5 (9)	LdEIta	4.17	0.17	5 (6)	HvE1a	7.50	0.15
6 (5)	HvE1a	4.04	0.21	5 (12)	HvE6s	7.50	0.15
7 (7)	HvE6a	3.67	0.19	7 (7)	PxE2	7.25	0.16
7 (10)	PxE2	3.67	0.12	8 (3)	Sf21	7.08	0.14
9 (1)	TNR[2]	3.54	0.00	9 (5)	LdEIta	6.83	0.14
10 (2)	Ag286	3.29	0.15	10 (7)	HvE6a	6.50	0.11
11 (3)	TND1	3.04	0.18	11 (1)	LdEp	6.42	0.17
12 (5)	HvE6s	2.92	0.19	12 (3)	TN368	6.25	0.18
AfMNPV				AfMNPV			
1 (12)	TN368	5.67	0.12	1 (11)	TNR[2]	9.67	0.11
2 (2)	Ag286	5.29	0.21	2 (2)	Ag286	9.17	0.14
3 (9)	LdEp	5.04	0.21	3 (8)	HvE6s	8.50	0.19
4 (8)	Sf21	4.67	0.12	4 (6)	HvT1	8.42	0.16
5 (6)	HvE1a	4.54	0.17	4 (10)	TND1	8.42	0.18
6 (4)	HvT1	4.42	0.12	6 (5)	HvE1a	8.33	0.16
6 (10)	LdEIta	4.42	0.12	7 (9)	HvE6a	7.92	0.16
8 (3)	HvE6s	4.17	0.17	8 (4)	Sf21	7.83	0.14
9 (7)	HvE6a	4.04	0.24	9 (3)	LdEp	7.75	0.13
10 (4)	TND1	3.79	0.21	10 (6)	LdEIta	7.50	0.15
11 (1)	TNR[2]	3.67	0.28	11 (12)	PxE2	6.92	0.14
12 (11)	PxE2	3.04	0.18	12 (1)	TN368	6.75	0.13
AgMNPV				AgMNPV			
1 (1)	Ag286	5.92	0.17	1 (1)	Ag286	8.67	0.22
2 (7)	LdEp	5.67	0.12	2 (5)	HvE1a	7.75	0.16
3 (4)	HvT1	3.67	0.25	3 (7)	PxE2	7.17	0.16
4 (5)	HvE6s	3.54	0.17	4 (3)	HvT1	7.00	0.13
5 (2)	HvE1a	3.42	0.25	5 (4)	HvE6s	6.75	0.14
6 (8)	Sf21	3.17	0.17	6 (8)	HvE6a	6.50	0.16
7 (3)	PxE2	2.67	0.25	7 (2)	LdEp	6.08	0.13
8 (6)	HvE6a	2.29	0.15	8 (6)	Sf21	5.83	0.20
9 (9)	LdEIta	2.04	0.18	9 (9)	LdEIta	5.75	0.17
10 (12)	TN368	1.42	0.12	10 (11)	TND1	5.42	0.00
11 (10)	TND1	1.17	0.17	11 (12)	TNR[2]	5.17	0.16
12 (11)	TNR[2]	0.67	0.12	12 (10)	TN368	4.50	0.16

extent seen in this study may be greater than expected. The general assumption is that the nucleocapsids in the two virus phenotypes are identical (Blissard, 1996). If this is true, then the infection process with the two phenotypes should be identical once the nucleocapsid reaches the nucleus of the cell. Therefore, the differences observed between BV and ODV infectivity must be due to differences in the early stages of the infection process—attachment to the cell membrane, penetration, and transport to the nucleus. These steps in infection are fairly well delineated with BV. Cell attachment is a receptor-mediated process in which the GP64 envelope fusion protein on the BV membrane is the ligand for the receptor on the cell plasma membrane (Hefferon et al., 1999). The virus envelope fuses with the resulting acidified endocytic vesicle, releasing the nucleocapsid into the cell cytoplasm followed by its transportation to the nuclear membrane.

The infection process with ODV is less well defined. As mentioned earlier, some evidence exists that attachment is also a receptor-mediated process (Horton and Burand, 1993) with the p74 ODV envelope protein (Faulkner et al., 1997; Slack et al., 2001) being a possible target for the cell receptor although my preliminary results mentioned previously with the AcMNPV p74-deletion mutant suggests this protein is not essential for the infection of cell cultures. In any case, instead of being an endosome-mediated process, the ODV envelope is believed to fuse to the cell membrane following attachment, which results in the release of the nucleocapsid into cell cytoplasm. While the nucleocapsid may be transported to the nucleus to initiate infection of the

cell, Granados and Lawler (1981) have suggested that at least some of the ODV nucleocapsids can bud from the cell, essentially forming OB-derived budded virus.

The trypsin effects observed with ODV on some cell lines in this study as well as previous work (Lynn, 1994) suggest proteases may play a role in the infection process of these viruses. Serine proteases (trypsin and chymotrypsin) are common digestive enzymes in insects (Applebaum, 1985) and, in fact, have been shown to play a roll in the pathogenicity of other insect pathogens, such as *Bacillus thuringiensis* (Oppert, 1999).

Further experiments need to be performed to determine if the results obtained with ODV in cell cultures are directly applicable to the infection process with OBs in larvae. If they prove to be, the degree of infectivity obtained with some cell/virus combinations, such as any of these viruses in LdEp cells or the AgMNPV in Ag286, should make these an effective tool for further study of this phenotype and much less difficult than similar studies in vivo. In particular, the importance of other ODV membrane-associated proteins can be examined. Also, the varied responses to trypsin treatment seen with different cell lines (i.e., TN-368 and LdEp) will provide a tool for delineating how this enzyme is enhancing ODV infections.

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